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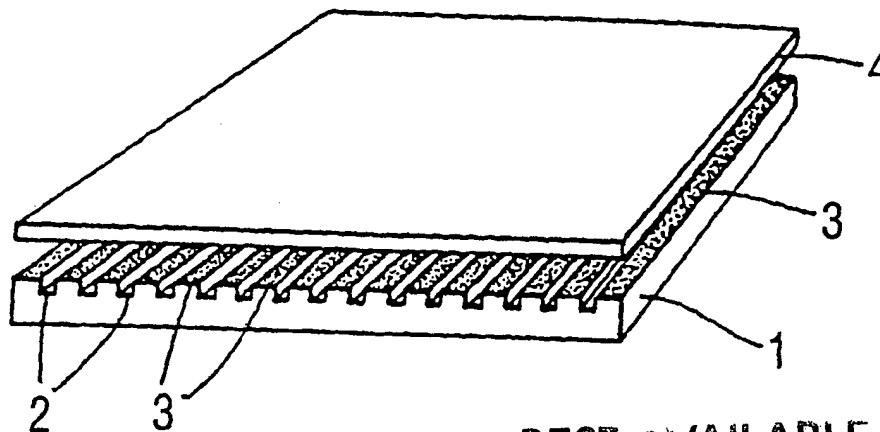
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(54) Title: TREATMENT OF SUBSTRATES



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(57) Abstract: A method of treating a substrate, which method comprises providing an electrolyte in contact with the substrate and an array of electrodes adjacent the surface and in contact with the electrolyte, and altering the potential of at least one electrode so as to generate an active redox product which modifies the substrate adjacent the at least one electrode, characterised in that the electrolyte is chosen such that the active redox product is quenchable by a second redox product. The method is particularly suitable for the step-wise chemical synthesis of oligomers such as oligonucleotides bound to a surface.

TREATMENT OF SUBSTRATES

This invention relates to the treatment of substrates using an electrochemical method. More particularly, this invention relates to a method of chemically modifying substrates using an electrochemical method.

Many devices require a pattern of a specific material on a surface. Semiconductor chips are a well-known example of such devices. A more recent example of such devices are DNA chips, which comprise an array of oligonucleotides bound to a solid surface (G. Ramsay, *Nature Biotechnology*, 1998, vol. 16, 40-44).

The properties of such devices depend on the nature and the pattern of the materials on the surface. Moreover, improvements to existing devices is driven on the one hand by demands for miniaturisation and on the other by the need for new types of surfaces which combine chemical and physical features. Hence, there is an increasing need for new methods of fabricating devices which require a pattern on its surface.

Several methods exist for the treatment of specific regions of surfaces. One method uses photolithographic technology. Specific regions of a surface are covered with a photolithographic mask and the exposed regions are modified by exposure to UV light. This method has been used widely in the manufacture of semiconductors in which apertures are created on the surface of a semiconductor wafer coated with a light-sensitive compound.

A photolithographic method has also been employed in the manufacture of DNA chips. In this method, oligonucleotides having photolabile protecting groups are bound to a solid surface. A region of the surface is covered with a photolithographic mask and the exposed regions of the surface irradiated with UV light. Hence, the photolabile protecting groups may be removed from the exposed region of the surface (G. Ramsay, *Nature Biotechnology*, 1998, vol. 16, 40-44).

WO93/22480 describes a method of treating a surface using an electrochemical method. In this method, there is provided an electrolyte overlying a surface and an array of electrodes adjacent the surface. By altering the potential of one or more electrodes of the array, the surface adjacent the one or more electrodes may be modified. The electrolyte employed is a solution of triethylamine and sulphuric acid in acetonitrile.

US 6,093,302 describes an electrochemical method of placing a material at a specific location on a substrate. The material is generated at an electrode and generally reacts with a substance proximate to the electrode. The use of a buffering or scavenging solution is described. The buffering or

scavenging solution is intended to improve the resolution of the substrate being treated by reacting with reagents that move away from the immediate vicinity of the electrodes. However, a bulk solution containing a buffering or scavenging substance has the disadvantage of quenching not only those reagents that diffuse away from specific electrodes, but also reagents which are intended to react at a substrate adjacent a specific electrode.

Schuster *et al.*, *Science*, 2000, 289, 98-101 describes another method of improving the resolution of an electrochemical method of treating a surface. Schuster employs a sequence of complicated current pulses to limit diffusion time.

It is an object of the present invention to provide an improved method of modifying a substrate using electrochemical means. In particular, it is an object of the present invention to provide a method of modifying a substrate with improved resolution.

Accordingly, the present invention provides a method of controlling the diffusion of a first redox product generated by a first electrode comprising generating a second redox product by a second electrode in proximity to said first electrode, the first and second electrodes being in contact with an electrolyte, wherein said electrolyte is such that the first redox product is quenchable by the second redox product.

Preferably, the second electrode is a counter electrode.

Preferably, the first redox product is an active redox product, which may be used for modifying a substrate adjacent the electrode.

Hence, in another aspect, the present invention provides a method of treating a substrate, which method comprises providing an electrolyte in contact with the substrate and one or more electrodes adjacent the substrate and in contact with the electrolyte, and altering the potential of at least one electrode so as to generate an active redox product which modifies the substrate adjacent the at least one electrode, characterised in that the electrolyte is such that the active redox product is quenchable by a second redox product.

By quenchable, it is meant that the second redox product is capable of reacting with the first redox product and modifying its reactivity so that the first redox product does not react in the same manner as it would in its original form. When the first redox product is an active redox product, the reaction between the active redox product and the second redox product will prevent the active redox product from modifying a substrate. For example, in the case where the active redox product is an acid, the

second redox product may be a base. The reaction between the acid and the base quenches the acid and prevents it from modifying a substrate.

5 In a preferred embodiment, the quenching reaction will regenerate one or more of the substances in the electrolyte.

By active redox product, it is meant any oxidation or reduction product which is capable of modifying a substrate. The active redox product may be generated directly by oxidation or reduction of a substance in the electrolyte. Alternatively, the active redox product may be generated indirectly
10 by oxidation or reduction of a substance in the electrolyte followed by one or more subsequent reactions with other substance(s) in the electrolyte.

Generally, the active redox product is generated at the surface of an electrode. The active redox product may then modify a substrate adjacent thereto. An acid is a preferred example of an active
15 redox product. An acid may be involved in many types of reaction on a substrate, for example eliminations, substitutions, rearrangements and chemical etching. Preferably, when the active redox product is an acid, the acid is used to remove an acid labile protecting group from a substrate.

Acid labile protecting groups are well known to a person skilled in the art and include, for example,
20 acetals (e.g. methoxymethyl, methylthiomethyl, (2-methoxyethoxy)methyl, benzoyloxymethyl, β -(trimethylsilyl)ethoxymethyl, tetrahydropyranyl, benzylidene, isopropylidene, cyclohexylidene, cyclopentylidene), esters (e.g. benzoyl, benzoyloxycarbonyl, tert-butoxycarbonyl), ethers (e.g. trityl, dimethoxytrityl, tert-butyl) and silyl ethers (e.g. tert-butyldimethylsilyl, trimethylsilyl, triethylsilyl). Preferably, the acid labile protecting group is a trityl or dimethoxytrityl (DMT) ether, which are
25 protecting groups commonly used in the synthesis of oligonucleotides.

Likewise, the active redox product may be a base. Bases may be involved in many types of reaction on a substrate. For example, a base may be used to remove a base-labile protecting group.

30 Base-labile protecting groups are well known to a person skilled in the art and include, for example, 9-fluorenylmethoxycarbonyl (Fmoc) and cyanoethyl groups.

Radicals are another example of an active redox product. Radicals may be used to initiate radical reactions on a substrate. Electrochemical methods for generating radicals will be well known to the
35 skilled person. One commonly used method for electrochemically generating a radical is the oxidation of carboxylate anions.

Halogens are another example of an active redox product. Halogens may be used in, for example oxidation reactions or addition reactions on a substrate. Halogens may be produced electrochemically by oxidation of the corresponding halide ion.

- 5 These and other examples of active redox products will be readily apparent to the skilled person.

The method of the present invention is used to treat a substrate. As used herein, the term substrate refers to any material or substance which is adjacent the electrode(s) and which may be modified by the active redox product. The substrate may be separate from the electrode(s), in which case the
10 substrate is placed adjacent the electrodes and then removed from the vicinity of the electrode(s) after the redox reaction has taken place. Alternatively, the substrate(s) may be attached to the electrode(s) themselves or attached to the same surface as the electrode(s). If desired, the substrate(s) may be cleaved from the electrode(s), or the same surface, after the redox reaction has taken place.

- 15 Hence, in one embodiment, the substrate is the surface of a material which is separate from and adjacent the electrode(s). Thus, the substrate may be the surface of a glass, plastics, solid fibre matrix, metal, semiconductor or gel material. The surface of this material may be modified directly by the redox reaction. Moreover, in this embodiment, the surface of the material may have substances attached thereto. Organic compounds, for example, may be attached to (and optionally
20 cleaved from) the surface of a material by known methods. Thus, substances attached to the surface of the material may be modified by the redox reaction.

In another embodiment, the substrate is a substance attached to the same surface as electrode(s), or a substance attached to the electrode(s) themselves, via a linker group. US 6,093,302 describes the
25 latter of these arrangements wherein the substrate is attached to the electrode(s) via linker groups.

The method of the present invention is similar to the method described in WO93/22480. However, the method of the present invention differs in the choice of electrolyte. WO93/2240 employs an electrolyte which is triethylamine and sulphuric acid in acetonitrile. The present invention uses an
30 electrolyte in which the active redox product is quenchable by at least one other redox product. An advantage of this electrolyte is that it is possible to confine precisely an active redox product to the region immediately surrounding the electrode by which it was generated.

In the method described in WO93/22480, the confinement of an acid at a specific region is controlled
35 by variation of the electrode potential. However, the present inventors have found that after prolonged electrolysis, the acid is unconfined when the electrolyte is triethylamine and sulphuric acid in acetonitrile. Poor confinement of acid leads to poor resolution of the substrate being treated. For example, protons which diffuse away from the immediate vicinity of the anode may react at the

substrate in the zone between electrodes. The adventitious reaction of diffused protons in this way is undesirable from the point of view of obtaining high resolution patterned substrates. By choosing an electrolyte in accordance with the present invention, the problems of the prior art electrolyte may be avoided. It is an important feature of the present invention that the electrolyte is chosen such that the active redox product is quenchable by at least one other redox product.

The skilled person will be aware of many examples of electrolytes which produce an active redox product that is quenchable by another redox product.

10 An example of such an electrolyte is a combination of I^- and $S_4O_6^{2-}$. Oxidation of iodide at the anode produces iodine (an active redox product), while reduction of $S_4O_6^{2-}$ at the cathode produces $S_2O_3^{2-}$, which may quench the iodine generated at the anode. The reactions in the electrolyte may be represented as follows:

15 Anode: $2I^- - 2e^- \rightarrow I_2$

Cathode: $S_4O_6^{2-} + 2e^- \rightarrow 2S_2O_3^{2-}$

Iodine is quenched by the reaction: $2S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2I^-$

20 Preferably, the active redox product is an acid and the quenching redox product is an anion, preferably an organic radical anion. Usually, the acid is generated at the anode by oxidation of an alcohol, which may be any aliphatic or aromatic alcohol. In such electrolytes, the quenching anion is usually generated at the cathode by reduction of a suitable substance. Many substances may be reduced at the cathode to produce an anion, which may quench the acid formed at the anode. For example, dissolved molecular oxygen may be reduced at the cathode, thereby generating O_2^- and/or O_2^{2-} .

30 An example of an electrolyte that produces suitable redox products is a combination of a ketone and a corresponding alcohol. Oxidation of the alcohol at the anode produces a proton (the active redox product), while reduction of the ketone at the cathode produces a radical anion which may quench the proton generated at the anode.

The reactions in the electrolyte may be represented as follows:

Anode: $R^1CH(OH)R^2 \rightarrow R^1C(O)R^2 + 2H^+ + 2e^-$

35 Cathode: $R^1C(O)R^2 + e^- \rightarrow [R^1C(O)R^2]^-$

wherein:

R^1 and R^2 are independently selected from optionally substituted C_1 to C_{15} hydrocarbyl wherein up to three C atoms may optionally be replaced by N, O and/or S atoms; or

R^1 and R^2 together form an optionally substituted C_1 to C_{15} cyclohydrocarbylene wherein up to three C atoms may optionally be replaced by N, O and/or S atoms.

5

Preferably, R^1 and R^2 are independently selected from optionally substituted C_{1-8} alkyl, C_{3-8} cycloalkyl or phenyl groups.

10 The term "hydrocarbyl" is used herein to refer to monovalent groups consisting of carbon and hydrogen. Hydrocarbyl groups thus include alkyl, alkenyl and alkynyl groups (in both straight and branched chain forms), cycloalkyl (including polycycloalkyl), cycloalkenyl and aryl groups, and combinations of the foregoing, such as alkylcycloalkyl, alkylpolycycloalkyl, alkylaryl, alkenylaryl, alkynylaryl, cycloalkylaryl and cycloalkenylaryl groups.

15 The term "hydrocarbylene" is used herein to refer to divalent groups consisting of carbon and hydrogen. Cyclohydrocarbylene groups thus include cycloalkylene, cycloalkenylene and arylene groups.

The term "aryl" is used herein to refer to an aromatic group, such as phenyl, naphthyl or anthracyl.
20 Alternatively, when an aryl group has carbon atoms replaced by O, N and/or S, the term aryl refers to a heteroaromatic group, such as pyridyl, pyrrolyl, thienyl, furanyl imidazolyl, triazolyl, quinolinyl, isoquinolinyl, oxazolyl or isoxazolyl.

Where reference is made herein to optionally substituted groups, the substituents are preferably
25 selected from C_1 to C_6 alkyl, C_1 to C_6 alkoxy, thio, C_1 to C_6 alkylthio, carboxy, carboxy(C_1 to C_6)alkyl, formyl, C_1 to C_6 alkylcarbonyl, C_1 to C_6 alkylcarbonylalkoxy, nitro, trihalomethyl, hydroxy, C_1 to C_6 alkylhydroxy, hydroxy(C_1 to C_6)alkyl, amino, C_1 to C_6 alkylamino, di(C_1 to C_6 alkyl)amino, aminocarboxy, C_1 to C_6 alkylaminocarboxy, di(C_1 to C_6 alkyl)aminocarboxy, aminocarboxy(C_1 to C_6)alkyl, C_1 to C_6 alkylaminocarboxy(C_1 to C_6)alkyl, di(C_1 to C_6 alkyl)aminocarboxy(C_1 to C_6)alkyl,
30 C_1 to C_6 alkylcarbonylamino, C_5 to C_8 cycloalkyl, C_5 to C_8 cycloalkyl(C_1 to C_6)alkyl, C_1 to C_6 alkylcarbonyl(C_1 to C_6 alkyl)amino, halo, C_1 to C_6 alkylhalo, sulphamoyl, tetrazolyl and cyano.

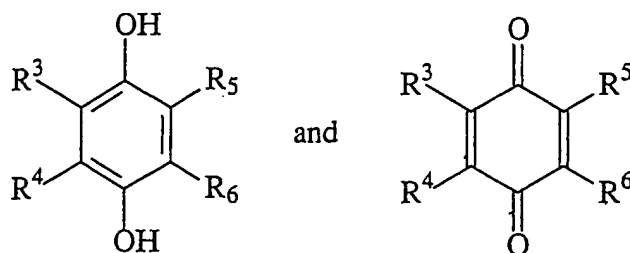
As used herein, "halo" or "halogen" refers to iodine, bromine, chlorine or fluorine.

35 The nature of R^1 and R^2 may be varied to change the redox characteristics of the electrolyte. For example, the introduction of substituents on R^1 and R^2 may change the potential at which oxidation or reduction occurs.

Preferred examples of ketone/alcohol electrolytes are 2-propanone/iso-propanol and benzophenone/benzhydrol, in a suitable organic solvent.

Another example of a suitable electrolyte is benzoquinone/hydroquinone and derivatives thereof.

- 5 Such electrolytes may be a combination of:



wherein R^3 , R^4 , R^5 and R^6 are independently selected from:

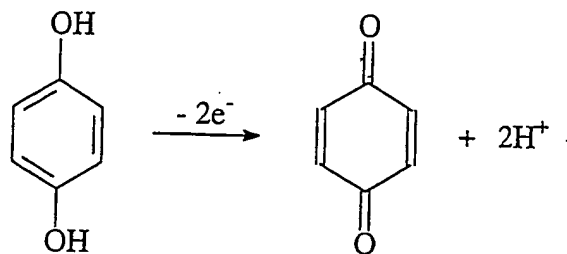
- 10 hydrogen, halo, nitro, hydroxyl, thio, nitro, amino,
optionally substituted C_1 to C_{15} hydrocarbyl wherein up to three C atoms may optionally be replaced by N, O and/or S atoms; or
 R^3 and R^4 and/or R^5 and R^6 together form an optionally substituted C_1 to C_{15} cyclohydrocarbylene wherein up to three C atoms may optionally be replaced by N, O and/or S atoms.

15

Preferably, R^3 , R^4 , R^5 and R^6 are independently selected from hydrogen, optionally substituted C_{1-8} alkyl or R^3/R^4 and R^5/R^6 together form an optionally substituted C_5 - C_{12} arylene group, such as phenylene.

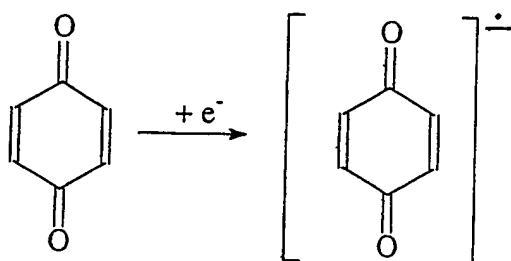
- 20 The nature of R^3 , R^4 , R^5 and R^6 may be varied to change the redox characteristics of the electrolyte, for example to alter the precise potential at which oxidation or reduction occurs. Preferred examples of electrolytes based on benzoquinone/hydroquinone derivatives are anthraquinone/anthraquinol and duroquinone/duroquinol, in a suitable organic solvent.
- 25 In a preferred embodiment, the electrolyte comprises a mixture of benzoquinone and hydroquinone in acetonitrile. This mixture provides an active redox product which is a hydrogen ion. The hydrogen ions (protons) are quenchable by a benzoquinone radical anion.

Specifically, hydroquinone is oxidised at the anode to produce benzoquinone and protons:

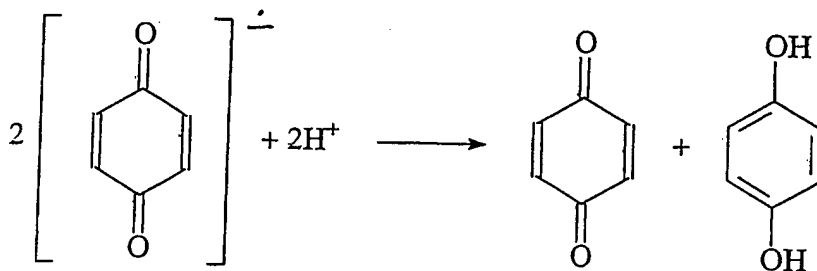


The protons liberated by the oxidation of hydroquinone are mostly localised at the anode and may modify a substrate adjacent thereto. For example, the protons may deprotect a substrate bearing an acid labile protecting group.

Benzoquinone is reduced at the cathode to produce a benzoquinone radical anion:



The benzoquinone radical anion is a relatively stable species in solvents such as acetonitrile. This radical anion quenches any adventitious protons which escape from the immediate vicinity of the anode, in accordance with the following reaction:



In this way, the resolution of a region of a substrate being treated may be improved by localising the active redox product generated at an electrode, for example, a proton generated at the anode.

The electrolytes used in the present invention may comprise any suitable solvent, such as water, THF (tetrahydrofuran), methanol, ethanol, DMF (dimethylformamide), dichloromethane, diethyl ether, DMSO (dimethylsulfoxide) or acetonitrile. The skilled person will appreciate that the choice of solvent may influence the kinetics or equilibrium of the redox reactions at the electrodes and/or the quenching reaction. The solvent may affect the reactivity of a species in solution by, for example,

complex formation, hydrogen bonding, dipole-dipole interactions or charge delocalisation. Preferably, the solvent is an aprotic solvent which is able to stabilise a radical anion. Examples of aprotic solvents are dichloromethane, DMF, DMSO, acetonitrile and THF. More preferably, the solvent is acetonitrile.

5

In a preferred embodiment, the electrolyte additionally comprises a conductivity enhancer. A conductivity enhancer is a substance which increases the conductivity of the electrolyte. It is desirable to increase the conductivity of the electrolyte so that electrolysis may be performed at lower voltages than in the absence of a conductivity enhancer. Any ionic substance which is soluble in the electrolyte is suitable for this purpose. For example, when the electrolyte comprises an organic solvent such as acetonitrile, a suitable conductivity enhancer may be a tetra(C₁₋₈ alkyl) ammonium salt, such as tetrabutylammonium hexafluorophosphate.

10

The skilled person will appreciate that a salt in the electrolyte may have effects, other than merely increasing the conductivity of the electrolyte. A salt may affect the kinetics or equilibrium of the quenching reaction and/or the redox reactions at the electrodes. The presence of salt is known to influence electrostatic interactions between charged species in solution. This, in turn, may affect reactivity. For example, when the electrolyte is hydroquinone/benzoquinone in acetonitrile, the addition of tetrabutylammonium hexafluorophosphate was found to modify the extent of the quenching reaction, as well as increase conductivity.

15

20

The method of the present invention may be performed using an apparatus as described in WO93/22480. The apparatus described in WO93/22480 comprises an array of electrodes spaced apart on an insulating surface. The electrodes are deposits of platinum, provided with electrical connecting means for altering their potentials.

25

However, it has been found that the method of the present invention is preferably performed using iridium electrodes. Hence, the present invention provides an array of electrodes, suitable for use in the method described herein, comprising a block of electrically insulating material having a surface, and deposits of iridium spaced apart in an array on the surface, each deposit being provided with electrical connecting means for altering its potential.

30

An advantage of using iridium is that it is highly conductive and chemically inert. Moreover, iridium does not suffer from degradation at the high electrical potentials which may be employed using the method of the present invention. Previously, platinum had been used as the electrode material. However, it was found that platinum does not adhere well to materials such as silicon wafers, especially at high electrode potentials. The internal quenching reaction described herein allows the

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prolonged use of high electrode potentials without significant loss of resolution at the substrate being treated. The use of high electrode potentials necessitated a change in electrode design.

5 A number of metals were tested for their suitability as electrodes including aluminium, silver and gold. However, the present inventors found, surprisingly, that iridium is an excellent choice of material for the electrodes. Iridium was found not to suffer from degradation during electrolysis and adheres well to materials such as oxidized silicon wafers.

10 The block of material on which the array of electrodes is formed may be made from any suitable material such as an insoluble polymer, ceramic oxides (e.g. alumina) or oxidized silicon wafers. Preferably, the material is an oxidized silicon wafer.

The array of iridium electrodes may be produced using any suitable method. In a preferred embodiment, the array of electrodes is made by a process comprising the steps of:

- 15 (i) providing a silicon wafer having a layer of silicon dioxide on the surface thereof;
(ii) depositing iridium in a spaced apart array on the silicon dioxide surface; and
(iii) annealing the iridium in air at a temperature in the range of 200-500°C.

20 In a typical procedure, a positive organic photoresist is applied to a silicon dioxide layer on a silicon wafer. The photoresist is exposed to UV light through a suitable photomask, revealing areas of silicon dioxide. Iridium metal is deposited on the surface of the material using an electron-beam gun. Removal of the photoresist layer then reveals the array of electrodes. Finally, the iridium electrodes are annealed in air to promote adhesion to the wafer surface. Typically, the iridium is annealed at about 350°C for a period of 15 mins to 3 hours, preferably about 1 hour.

25 The annealing step is important for adhesion of the iridium to silicon dioxide. The effect of annealing at a temperature of about 350°C is surprising, given that iridium has a melting point of 2545°C. It has been found that, even with a 50 nm layer of iridium, the annealed electrodes can withstand scratching with a steel scalpel blade. Moreover, the iridium electrodes are able to withstand the harsh chemical
30 environment, high potentials and high current which may be used in the method of the present invention.

35 Preferably, the electrodes are an array of parallel lines spaced apart by less than 0.5 mm. Preferably, the electrodes are spaced apart by 0.1-200 microns, more preferably, 1-100 microns and more preferably 10-60 microns.

Preferably, one or more of the electrodes is used as a counter-electrode. Preferably, the substrate to be modified does not form either an electrode or a counter-electrode, in contrast to conventional

methods of treating substrates electrochemically. Hence, the method of the present invention may be similar to the method described in WO93/22480. Further, the substrate to be treated may be an electrically insulating surface.

- 5 In a preferred embodiment, the present invention provides a method of performing several treatments in sequence. Thus, the electrodes of the array are preferably connected up so that each treatment is performed by altering the potential of a chosen set of one or more electrodes of the array.

- 10 Preferably, in the method of the present invention, the substrate to be treated comprises a substance bound to a solid surface. The solid surface may be the surface bearing the electrodes. Preferably, the solid surface is a different surface adjacent the electrodes. The active redox product may be used to produce a chemical modification of the solid bound substance. The skilled person can conceive of many active redox products and corresponding chemical modifications. In a preferred embodiment, the substrate to be treated comprises a substance having an acid-labile protecting group. In this
15 preferred embodiment, the treatment is performed by connecting at least one electrode of the array as an anode at a potential which generates an acid in the electrolyte. The acid thus generated will then remove an acid-labile protecting group from the substance bound to the surface in the region adjacent the anode.

- 20 However, it will be appreciated that the active redox product may be involved in a variety of chemical reactions at a substrate. One potential application is in the electrochemical micromachining technology described by Schuster *et al.*, *Science*, 2000, 289, 98-101. The tool described by Schuster may be adapted by using an electrolyte in accordance with the present invention and surrounding the probe tip with a ring of a counter electrode to prevent diffusion of the redox product. Thus, the
25 present invention may be applied to existing nanoscale patterning techniques. An acid, for example, may be used in etching or nanofabrication applications, which require removal of a small amount of a material from a surface.

- Alternatively, an acid may be involved in any organic or inorganic reaction promoted by acid. The
30 skilled person will be aware of a very large number of potential reactions which could be adapted for use with present invention. Examples of organic reactions include epoxide openings, additions to multiple bonds, rearrangements, substitutions (e.g. S_N1 substitution of a tertiary alcohol), eliminations, formation of enols with subsequent reactions of the enol, and simple protonation of organic acid salts.

35

Equally, when the active redox product is a halogen, the halogen may be involved in mild oxidations, bleaching a substrate or halogenations. The active redox product may also be a halide ion, which may be used in substitution reactions.

The method of the present invention may also be used in the synthesis of libraries of small organic compounds bound to a surface (see, for example, Schreiber, *Science* 2000, 287, 1964-1969). Libraries of small organic compounds are important in the field of drug discovery. The range of reactions to which the present method may be applied means it is ideally suited to the synthesis of such libraries.

Preferably, the method of the present invention is used in the stepwise chemical synthesis of oligomers, such as oligonucleotides, polysaccharides and proteins. More preferably, the method of the present invention is used in the synthesis of oligonucleotides.

A method of synthesising a set of oligomers may comprise the steps of:

- (a) providing a substrate having attached thereto an array of substances having a protecting group, an electrolyte in contact with the substrate and an array of electrodes adjacent the substrate and in contact with the electrolyte;
- (b) selectively altering the potential of one or more of the electrodes so as to generate an active redox product which removes the protecting group from selected substances;
- (c) coupling a protected monomer with the deprotected substances formed in step (b); and
- (d) repeating steps (b) and (c), while varying the one or more electrodes selected in step in step (b), so as to synthesise a set of oligomers;

characterized in that the electrolyte is chosen such that the active redox product is quenchable by at least one other redox product.

When the above-described method is used in the synthesis of oligonucleotides, the active redox product is preferably a proton and the protecting group is preferably an acid labile protecting group, such as a trityl or dimethoxytrityl (DMT) group which protects a furanyl hydroxyl group. The skilled person will appreciate that this method is particularly suited to the combinatorial synthesis of DNA chips, as described in WO93/22480.

The above-described method may also be used in the synthesis of peptides. For example, peptides may be synthesised by sequential removal of *t*-butyloxycarbonyl (Boc) protecting groups from nitrogen atoms using protons generated at the anode. Other oligomer syntheses will be readily apparent to a person skilled in the art.

The present invention will now be described in more detail with reference to the following Figures in which:-

Figure 1 shows an apparatus suitable for carrying out the method of the present invention;

Figure 2 shows schematically how a selected region of a substrate may be modified;

5 Figure 3 shows the effect of varying the time of electrolysis; and

Figure 4 shows the effect of removing a cathode from the array of electrodes.

Referring to Figure 1, an array of electrodes is based on an oxidized high-resistivity silicon wafer (1),
10 the upper surface of which has a layer of iridium deposited thereon. Gaps (2) are formed in the
iridium layer on the silicon wafer using positive-resist photolithography, resulting in an array of
parallel electrodes (3). The width of each electrode and of each gap is approximately 40 microns. A
silicon wafer (4) is placed over the array of electrodes. The silicon wafer is modified to present a
DMT-protected nucleoside at its surface.

15

Referring to Figure 2, part of an array of electrodes and a substrate are shown. The central electrode
is an anode and the two other electrodes are cathodes. An electrolyte comprising benzoquinone and
hydroquinone in acetonitrile is in contact with the electrodes and the substrate to be treated. At the
anode, hydroquinone is oxidized, thus generating benzoquinone and protons. The majority of protons
20 are confined at a region of the substrate adjacent the anode. Thus, the confined protons remove DMT
groups from a protected nucleotide moiety bound to the substrate. However, some of the protons are
able to diffuse into the zone between the anode and the cathode.

At the cathode, a benzoquinone radical anion is produced by reduction of benzoquinone. The
25 benzoquinone radical anion is a relatively stable species and is able to diffuse into the zone between
the anode and the cathode. The benzoquinone radical anion quenches any protons which have
diffused into this zone, thereby producing hydroquinone and benzoquinone as shown. Thus, the
diffused protons are prevented from reacting at a region of the substrate which is not adjacent an
anode. By preventing protons from reacting adventitiously in regions between electrodes, the
30 resolution of the patterned substrate is improved.

Figure 2 also shows subsequent treatment of the substrate following the above-described detritylation
from a specific region of the substrate. The free hydroxyl groups are acetylated under standard
conditions and the remainder of the DMT groups removed. The resulting free hydroxyl groups are
35 treated with a fluorescent dye (Cy5 phosphoramidite), which allows imaging of the substrate by
confocal microscopy. Thus, the resolution of the initial detritylation step may be conveniently
analysed. However, it will be readily apparent that an oligomer may be synthesised on a selected
region of the substrate using the above-described methodology.

Referring to Figure 3, the effect of varying the time of electrolysis, at a fixed potential of 1.33 V, is shown using confocal microscopy. In this Figure, light regions show fluorescence in regions of the substrate in which no DMT groups have been removed during electrolysis. The remaining DMT groups are subsequently replaced with a fluorescent Cy5 dye. The light regions are generally adjacent cathodes. Dark regions are those in which the DMT groups have been removed during electrolysis. The resulting free hydroxyl groups are acetylated with a non-fluorescent acetyl group. The dark regions are generally adjacent anodes.

Figure 3 shows that after 2.0 s, the DMT groups are fully removed in the regions adjacent anodes. Moreover, the resolution of the patterned substrate is not changed after 80 s. There are sharply defined stripes corresponding to regions adjacent the anodes and cathodes. This demonstrates that the protons generated during electrolysis are strictly confined to regions adjacent the anodes, even after prolonged electrolysis.

Figures 4(a) and (b) show the dramatic effect of removing a cathode from the array of electrodes. The dark regions show regions in which DMT groups have been removed. The electrode potential is fixed at 1.33 V. Once the central cathode has been removed, protons generated at the anode are allowed to diffuse freely into the central region. This clearly demonstrates the confining effect of having a cathode generating a species which is able to quench protons produced at the anode.

The method of the present invention is described in more detail in the following Examples.

Experimental Section

Electrode Assembly

Conventional positive resist photolithography was used to produce iridium metal (50 nm thickness) electrodes on oxidized high-resistivity silicon wafers. The oxidized silicon wafers were coated with a positive photoresist layer and exposed to UV light through a photomask. The wafer was washed with deionized water, baked at 100°C for 20 minutes and "descummed" by reactive ion etching. The photomask was chosen to give an array of 96 parallel electrodes, about 7500 microns long and 40 microns wide. The gap in between adjacent electrodes was about 40 microns.

Iridium was deposited on the wafer by an electron-beam method. Iridium metal was placed in a crucible in a vacuum evaporator and two or three wafers were situated approximately 20 cm from the crucible in the vacuum evaporator. The wafers were coated with 50 nm iridium by pumping the

chamber to 3×10^{-6} Torr and heating the metal with an electron-beam gun set to 300 mA at 5kV for about 3 minutes.

5 The photoresist layer was then removed by placing the wafer in an ultrasonic acetone bath for 30 minutes, thus revealing an array of electrodes. The electrodes were annealed at 350 °C in air for about 1 hour to promote adhesion with the wafer substrate, and cleaned by reactive ion etching.

10 After the heat annealing and cleaning step, each electrode was individually connected by ultrasonic gold wire bonding to a printed circuit board, where digitally controlled "analog switch" integrated circuits activate electrodes chosen for a given deblocking step. Current was applied as multiple independent operational amplifier-controlled voltages sources. Parallel low-noise instrumentation amplifier feedback circuits continuously measured nanoamp-precision current at each of the electrodes. A computer programmed specially for this work controlled all voltages, timing, and
15 electrode switching, and collected current measurement data.

Solid Support Assembly

20 Polished silicon dioxide wafers were used as the patterned substrate supports. Before electrochemical patterning, the wafer surface was functionalized with a linker molecule to which the organic reagents were attached (Gray, D.E., CaseGreen, S.C., Fell, T.S., Dobson, P.J. & Southern, E.M. Ellipsometric and interferometric characterization of DNA probes immobilized on a combinatorial array. *Langmuir* 13, 2833-2842 (1997)). Wafers were placed in a vacuum furnace chamber, 19.1 l in volume, with an ampoule containing 5 ml glycidoxypopyltrimethoxysilane. After heating the furnace to 185 °C, the
25 ampule was heated to 205 °C and the chamber evacuated to 25-30 mBar. After approximately 2.5 ml of the silane had evaporated, the chamber was allowed to cool under vacuum (10^{-3} Torr). A "linker molecule" was attached by immersing the glycidoxypopyltrimethoxysilane-derivatized wafers in a 100% solution of polyethylene glycol containing a trace of sulfuric acid. DMT-containing phosphoramidite was then covalently attached to the free hydroxyl on the polyethylene glycol by
30 conventional oligonucleotide synthesis techniques (Beaucage, S.L. & Iyer, R.P. Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach. *Tetrahedron* 48, 2223-2311 (1992)). The wafer substrate thus prepared was cut into 1 cm squares for use in patterning.

Example 1

35

The electrode array (as prepared above) was placed at a distance of 20 microns from the solid support. The solid support was prepared as described above, with a thymidine phosphoramidite

attached to the polyethylene glycol linker molecule. The thymidine phosphoramidite had its 5'-hydroxy group protected by a DMT group.

5 A solution of electrolyte (25 mM hydroquinone/25 mM quinone/25 mM tetrabutylammonium hexafluorophosphate in anhydrous acetonitrile) was introduced in the cavity between the electrode array and the solid support. Selected anodes were then set at 1.33 V with respect to the cathodes and the voltage maintained for 0.2 to 80 s, as shown in Figure 3.

10 Following electrolysis, the silicon wafer was washed with acetonitrile and acetylated with acetic anhydride using a standard method. Only the DMT-deprotected regions of the silicon wafer, having exposed hydroxyl groups, were acetylated in this step.

15 The DMT groups not removed by the electrochemical step were then removed by treating the whole substrate with a solution of dichloroacetic acid in dichloromethane. The hydroxyl groups thus exposed were coupled to Cy5, a fluorescent dye, using a standard phosphoramidate coupling method so that the pattern produced by the electrochemical generation of acid was revealed by observing the fluorescence of the Cy5 in a confocal microscope. This sequence of steps is shown in Figure 2.

20 Figure 3 shows the effect of increasing the electrolysis time at 1.33 V. Once a maximum band width is reached after about 2.0 s, the resolution of the substrate is maintained, even after electrolysis for 80.0 s.

Example 2

25 Example 1 was repeated exactly as described above, with the exception that a voltage of 1.33 V was maintained at selected anodes for 16 s. The dramatic effect of removing a cathode was investigated, as shown in Figures 4(a) and (b). With the central cathode removed, there is no control of diffused protons. The diffused protons are allowed to flood into the central region and are not localised around the anodes. This is evidenced by the central dark area in Figure 4(b), which contains no fluorescent groups.

Example 3

30 The general method described in Example 1 was used in the synthesis of a 17-mer oligomer on a solid support, with 16 electrochemically controlled DMT deprotection steps. The method used was 35 the same as Example 1, with the exception that the array of electrodes was placed at a distance of 40 microns from the surface of the substrate.

A uniform covering of a dimethoxytrityl (DMT) protected deoxyadenosine (dA) residue was coupled to the polyethylene glycol linker group on the solid support, using standard phosphoramidite coupling chemistry.

- 5 Following extensive washing with acetonitrile, the electrolyte used in Example 1 was introduced into the cavity between the electrode array and the solid support. A potential of 1.33 V was applied to a selected anode for 9 s to remove the DMT groups adjacent thereto. The anode was flanked by two cathodes.
- 10 Following further washing with acetonitrile, a DMT-protected nucleotide residue was coupled to the exposed hydroxy groups using a standard phosphoramidite coupling. The resulting trivalent phosphorus linkage was oxidized with iodine to yield a pentavalent phosphorus linkage and the whole silicon wafer was then washed with acetonitrile followed by dichloromethane. The standard coupling and oxidation steps used in the synthesis of oligonucleotides on solid supports are well
- 15 known in the art. (See, for example, *ABI Synthesizer Manual*, Section 2: "Chemistry for Automated DNA Synthesis").

The procedure was repeated, varying the DMT-protected nucleotide residue introduced in the phosphoramidite coupling step. Thus, an oligonucleotide was synthesized on the solid support.

20

- This procedure has been employed on an automated apparatus, controlled by a suitably programmed computer, in the synthesis of two 17-mer oligonucleotides: wild type "A" human hemoglobin mRNA and the corresponding "S" type sickle cell mutant mRNA. The 17-mers were built up on a defined strip of the solid support in excellent yields. The skilled person will readily appreciate that this
- 25 method may be used for the combinatorial synthesis of DNA chips, as described in WO93/22480.

It will, of course, be appreciated that this invention has been described by way of example only and that modifications of detail may be made within the scope of the invention.

CLAIMS

1. A method of controlling the diffusion of a first redox product generated by a first electrode comprising generating a second redox product by a second electrode in proximity to said first electrode, the first and second electrodes being in contact with an electrolyte, wherein said electrolyte is such that the first redox product is quenchable by the second redox product.

2. A method as claimed in claim 1 wherein the second electrode is a counter-electrode.

3. A method as claimed in claims 1 or 2 wherein the first redox product is an active redox product.

4. A method as claimed in claim 3 wherein the active redox product is used for modifying a substrate adjacent the electrode.

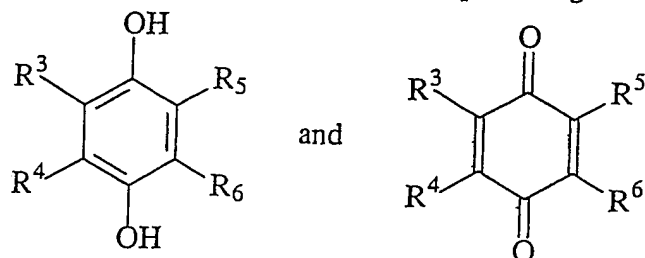
5. A method of treating a substrate, which method comprises providing an electrolyte in contact with the substrate and one or more electrodes adjacent the substrate and in contact with the electrolyte, and altering the potential of at least one electrode so as to generate an active redox product which modifies the substrate adjacent the at least one electrode, characterised in that the electrolyte is such that the active redox product is quenchable by a second redox product.

6. A method as claimed in claims 3 to 5 wherein the active redox product is a proton.

7. A method as claimed in any one of the preceding claims wherein the second redox product is an organic radical anion.

8. A method as claimed in any one of the preceding claims wherein the electrolyte is a solution of hydroquinone and benzoquinone, or derivatives thereof.

9. A method as claimed in any one of the preceding claims wherein the electrolyte is a solution of:



wherein R³, R⁴, R⁵ and R⁶ are independently selected from:

- hydrogen, halo, hydroxyl, thio, nitro, amino, optionally substituted C₁ to C₁₅ hydrocarbyl wherein up to three C atoms may optionally be replaced by N, O and/or S atoms; or
- R³ and R⁴ and/or R⁵ and R⁶ together form an optionally substituted C₁ to C₁₅ cyclohydrocarbylene wherein up to three C atoms may optionally be replaced by N, O and/or S atoms.

5

10. A method as claimed in any one of the preceding claims wherein the electrolyte is a solution of hydroquinone and benzoquinone in acetonitrile.

11. A method as claimed in any one of the preceding claims wherein the electrolyte further comprises a conductivity enhancer.

10 12. A method as claimed in claim 7 wherein the conductivity enhancer is a tetra(C₁₋₈ alkyl) ammonium salt.

13. A method as claimed in any one of claims 5 to 12 wherein one or more of the electrodes are used as counter-electrodes and the substrate to be modified does not form either an electrode or a counter-electrode.

15 14. A method as claimed in any one of the preceding claims wherein, for the purposes of performing several treatments in sequence, the electrodes of the array are connected up so that each treatment is performed by altering the potential of a chosen set of one or more of the electrodes of the array.

15. A method as claimed in any one of the preceding claims wherein the substrate comprises an
20 array of substances bound to a surface.

16. A method as claimed in claim 15 wherein the surface is a surface of an oxidized silicon wafer.

17. A method as claimed in claim 15 wherein the substances to be treated comprise an acid labile protecting group.

18. A method as claimed in claims 17 wherein each treatment is performed by connecting at least
25 one electrode of the array as an anode at a potential to remove an acid labile protecting group from a substance on the surface.

19. A method as claimed in any one of claims 14 to 18 wherein the or each treatment is performed in the course of a stepwise chemical synthesis of an oligomer.

20. A method of synthesising a set of oligomers comprising the steps of:

30 (a) providing a substrate having attached thereto an array of substances having a protecting group, an electrolyte in contact with the substrate and an array of electrodes adjacent the substrate and in contact with the electrolyte;

- (b) selectively altering the potential of one or more of the electrodes so as to generate an active redox product which removes the protecting group from selected substances;
- (c) coupling a protected monomer with the deprotected substances formed in step (b); and
- (d) repeating steps (b) and (c), while varying the one or more electrodes selected in step in
- 5 step (b), so as to synthesise a set of oligomers;
- characterized in that the electrolyte is chosen such that the active redox product is quenchable by at least one other redox product.

21. A method as claimed in claim 20 wherein the array of substances are attached to a surface and

10 the oligomer is synthesized on said surface.

22. A method as claimed in claims 20 or 21 wherein the oligomers are oligonucleotides.

23. A method as claimed in claims 20 to 22 wherein the active redox product is a proton and the

15 protecting groups are acid labile protecting groups.

24. A method as claimed in any one of claims 20 to 23 wherein the electrolyte is as defined in claims 8 to 12.

20 25. A method as claimed in any one of claims 20 to 24 wherein one or more of the electrodes of the array are used as counter electrodes.

26. An array of electrodes, suitable for use in the method of any one of the preceding claims, comprising a block of insulating material having a surface, and deposits of iridium spaced apart in an

25 array on the surface, each deposit being provided with electrical connecting means for altering its potential.

27. An array as claimed in claim 26 wherein the block of insulating material is an oxidized silicon wafer.

30

28. An array as claimed in claims 26 or 27 wherein the deposits of iridium are in the form of spaced apart parallel lines.

29. An array as claimed in claims 26 to 28 wherein said array is made by a process comprising the

35 steps of:

- (i) providing a silicon wafer having a layer of silicon dioxide on the surface thereof;
- (ii) depositing iridium in a spaced apart array on the silicon dioxide surface; and
- (iii) annealing the iridium in air at a temperature in the range of 200-500°C.

1/3

FIG. 1

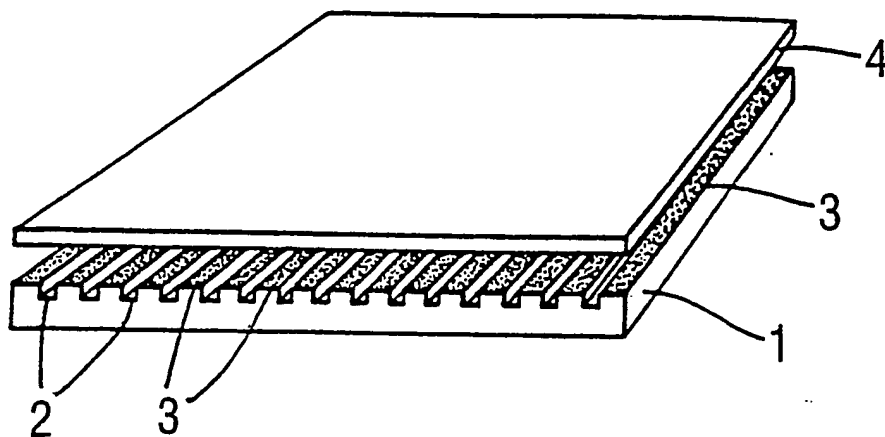


FIG. 4(a)

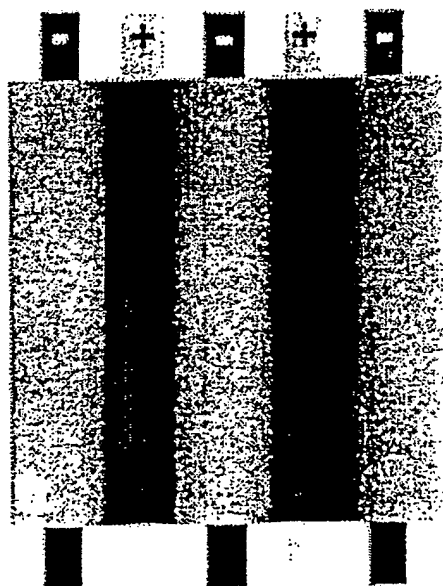
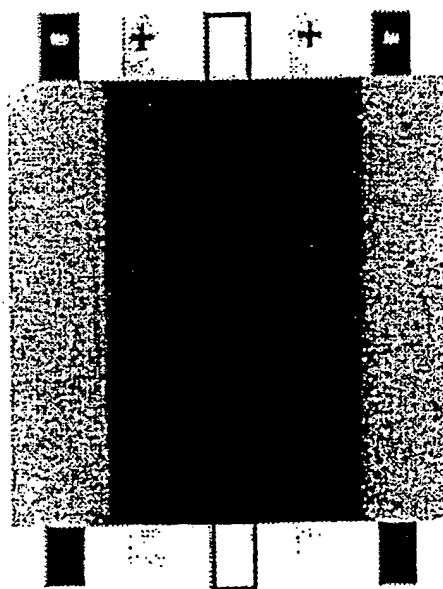


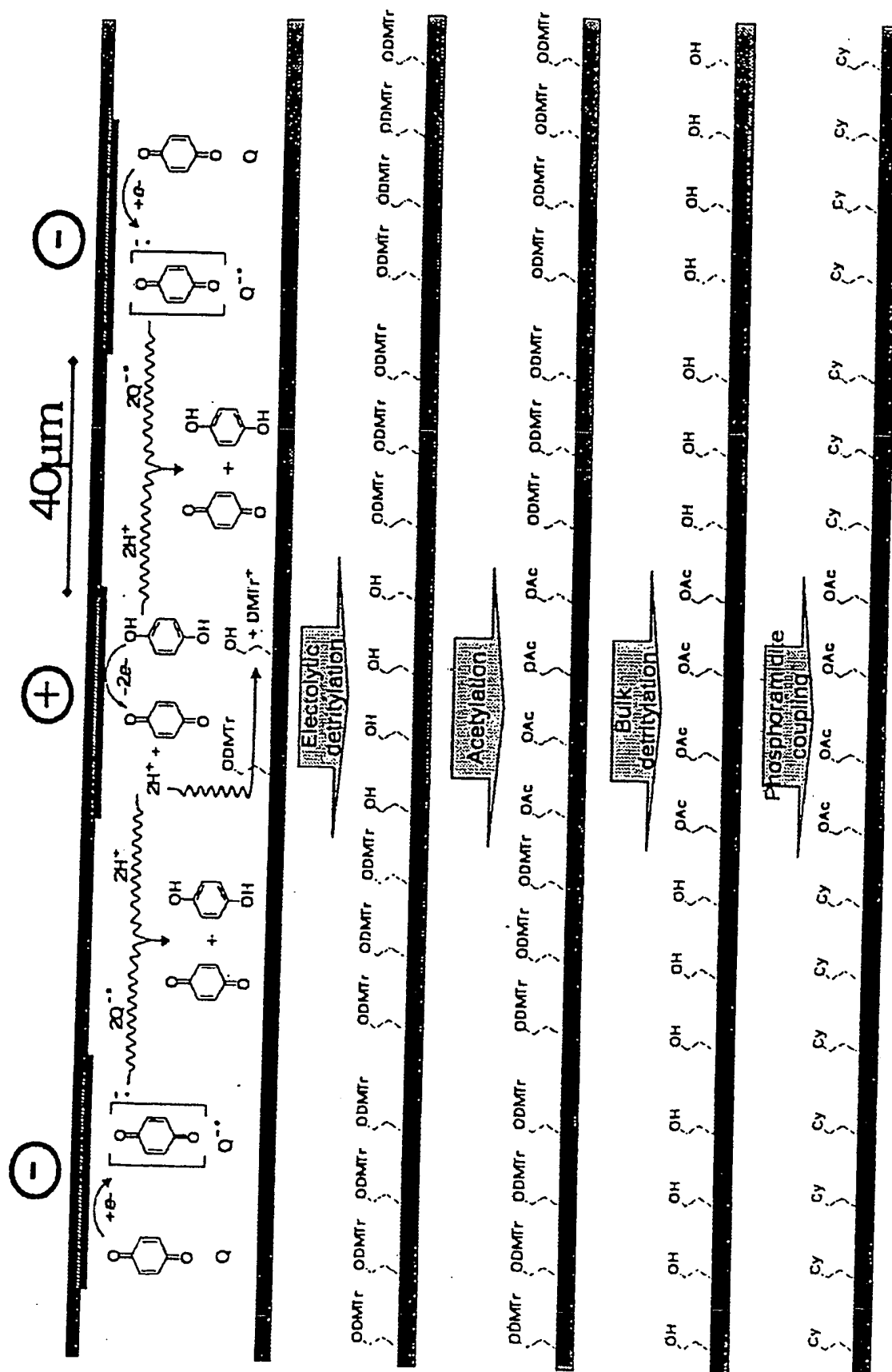
FIG. 4(b)



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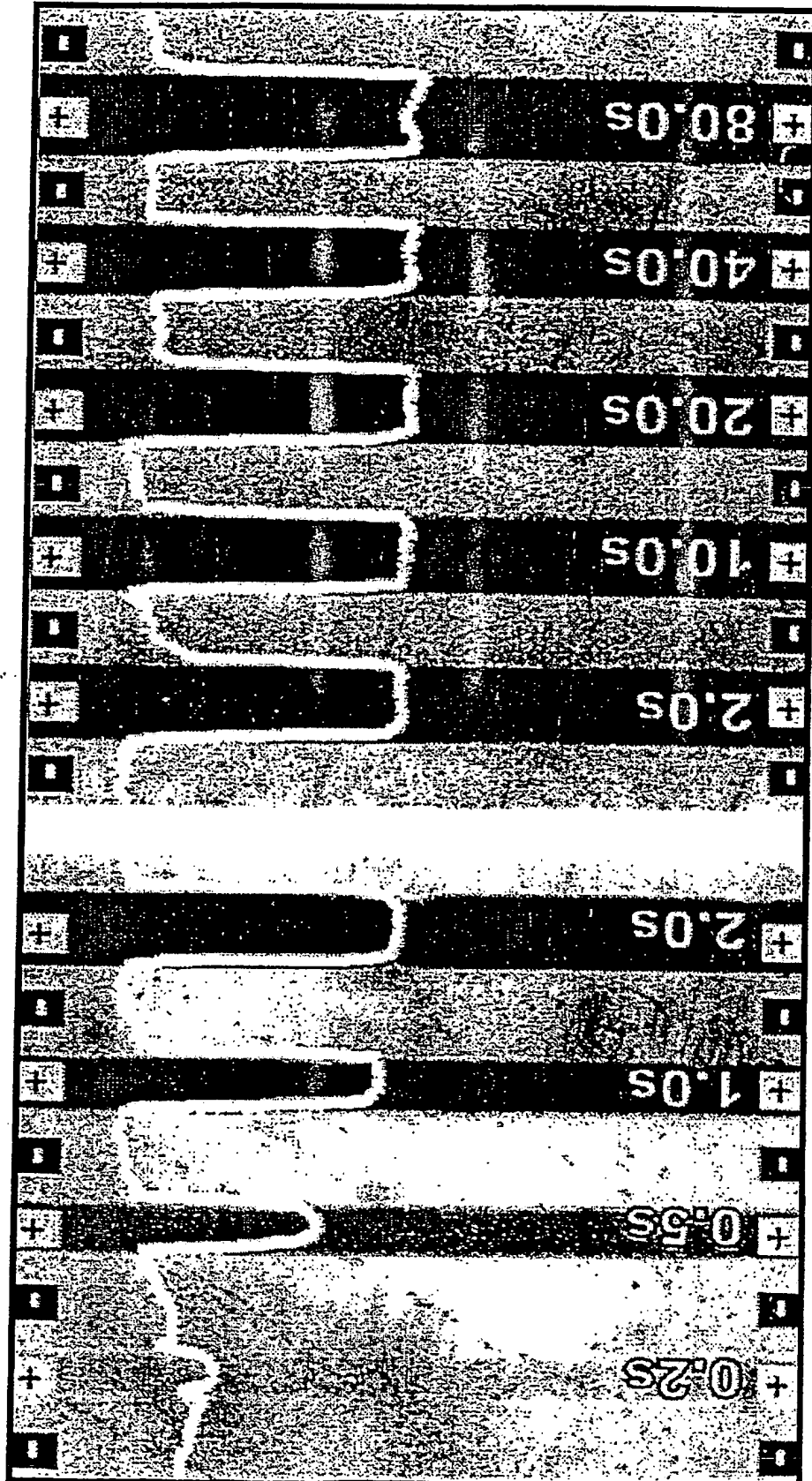
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FIG. 2



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FIG. 3



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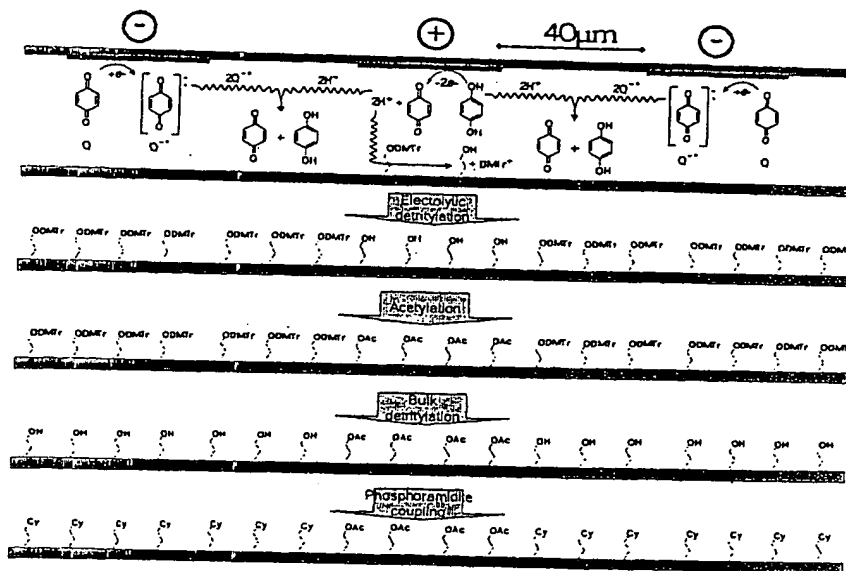
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(54) Title: **TREATMENT OF SUBSTRATES USING AN ELECTROCHEMICAL METHOD**



(57) Abstract: A method of treating a substrate, which method comprises providing an electrolyte in contact with the substrate and an array of electrodes adjacent the surface and in contact with the electrolyte, and altering the potential of at least one electrode so as to generate an active redox product which modifies the substrate adjacent the at least one electrode, characterised in that the electrolyte is chosen such that the active redox product is quenchable by a second redox product. The method is particularly suitable for the step-wise chemical synthesis of oligomers such as oligonucleotides bound to a surface.

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WO 03/020415 A3



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 02/03992

A. CLASSIFICATION OF SUBJECT MATTER
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 093 302 A (MONTGOMERY DONALD D) 25 July 2000 (2000-07-25) cited in the application abstract column 11, line 15 -column 12, line 33 column 12, line 34 -column 13, line 8 column 20, line 17 -column 22, line 17; figures 1-4,31,34,37 column 23, line 1 - line 8 column 26, line 10 -column 27, line 15; figures 6-8	1-29
A	WO 93 22480 A (ISIS INNOVATION ;SOUTHERN EDWIN (GB)) 11 November 1993 (1993-11-11) cited in the application the whole document --- -/--	1-25

☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/03992

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passage	Relevant to claim No.
X	SULLIVAN M G ET AL: "AUTOMATED ELECTROCHEMICAL ANALYSIS WITH COMBINATORIAL ELECTRODE ARRAYS" ANALYTICAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. COLUMBUS, US, vol. 71, no. 19, 1 October 1999 (1999-10-01), pages 4369-4375, XP001032132 ISSN: 0003-2700 page 4371, column 2 ---	1, 5, 7-9
X	US 5 922 183 A (RAUH R DAVID) 13 July 1999 (1999-07-13) abstract column 5, line 57 - line 60 column 6, line 1 - line 4 column 7, line 13 - line 18 ---	26-29
X	US 5 378 343 A (KOUNAVES SAMUEL P ET AL) 3 January 1995 (1995-01-03) column 3, line 58 - line 65; claims 1-3 ---	26-29
X	KOVACS G T A ET AL: "REGENERATION MICROELECTRODE ARRAY FOR PERIPHERAL NERVE RECORDING AND STIMULATION" IEEE TRANSACTIONS ON BIOMEDICAL ENGINEERING, IEEE INC. NEW YORK, US, vol. 39, no. 9, 1 September 1992 (1992-09-01), pages 893-902, XP000322966 ISSN: 0018-9294 the whole document ---	26-29
X	WO 97 34140 A (HINTSCHE RAINER ;FRAUNHOFER GES FORSCHUNG (DE); PAESCHKE MANFRED ()) 18 September 1997 (1997-09-18) page 9, line 24 - line 30; figure 1A page 3, line 15 - line 21 -----	26-29

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-25

Method of controlling diffusion.
Method of treating a substrate.
Method of synthesising a set of oligomers.

2. Claims: 26-29

Array of electrodes comprising iridium.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/03992

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 02/03992

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